

Relative potencies for barbiturate binding to the *Torpedo* acetylcholine receptor

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1 The structural requirements of an allosteric barbiturate binding site on acetylcholine receptor-rich membranes isolated from *Torpedo* electroplaques have been characterized by the ability of fourteen barbiturates to displace [¹⁴C]-amobarbitone binding.

2 The barbiturates could be grouped into two classes with ten barbiturates producing a strong inhibition of [¹⁴C]-amobarbitone binding (class one) and with four exerting minimal effects (class two).

3 Eight of the ten class one barbiturates displaced essentially all of the [¹⁴C]-amobarbitone from its binding site, while, at their respective aqueous solubility limits, two of these barbiturates (thiopentone and dimethylbutylbarbitone (DMBB)) inhibited [¹⁴C]-amobarbitone binding by nearly 80%. The apparent inhibition constants (K_i) for the class one barbiturates ranged from 13 μM for amobarbitone to 2.8 mM for barbitone with the other eight agents lying in the range 100–600 μM , and having the rank order pentobarbitone \approx secobarbitone > thiopentone > DMBB > butobarbitone \approx phenobarbitone > aprobarbitone > allylbarbitone.

4 By contrast, the class two barbiturates had minimal effects even at close to saturating concentrations. [¹⁴C]-amobarbitone binding was reduced slightly (<30%) by hexobarbitone, mephobarbitone and methohexitone and was enhanced slightly (<20%) by metharbitone.

5 All of the class two, but none of the class one barbiturates, were N-methylated.

Introduction

The molecular mechanisms underlying the various clinical actions of barbiturates remains unclear. Several studies suggest that barbiturates may exert some of their anti-convulsive, anxiolytic and anaesthetic actions by allosterically enhancing and/or inhibiting postsynaptic responses (Barker *et al.*, 1980; Ho & Harris, 1981; Willow & Johnston, 1983; MacDonald *et al.*, 1986). The most extensively studied of these possible sites has been in pathways using the inhibitory neurotransmitter, γ -aminobutyric acid (GABA) (Simmonds & Turner, 1987; Schwartz, 1988; Olsen, 1982). Studies have suggested the existence of a barbiturate binding site on the GABA receptor that appears to be capable of allosterically enhancing GABA binding. Less is known about the effect of barbiturates on excitatory postsynaptic responses. Several studies have focused on barbiturate-acetylcholine receptor interactions (reviewed in Richter & Holtman, 1982). Because nicotinic receptors in the central nervous system are not well characterized, electrophysiological studies have focused on the neuromuscular junction. Here, pharmacological studies show the ability of barbiturates to inhibit synaptic transmission parallels their anaesthetic potency, implying a nonspecific mechanism (Lee-Son *et al.*, 1975). However, mechanistic studies found barbiturates to inhibit transmission at the neuromuscular junction by selectively blocking open ion channels (Adams, 1976) in a manner most consistent with a mechanism involving an allosteric barbiturate site (Gage & McKinnon, 1985).

Because of the low density of nicotinic receptors in mammalian tissues, the question of the existence of specific barbiturate sites is best approached in other nicotinic systems, such as the *Torpedo* electroplaque. In acetylcholine receptor-rich membranes from this tissue, there is direct evidence for a stereoselective functional barbiturate binding site that is allosterically coupled to the acetylcholine binding site (Dodson *et al.*, 1987; Roth *et al.*, 1989). In this work we have extended our

studies to a wider range of barbiturates, obtaining a self-consistent and accurate set of data for fourteen barbiturates in order to explore the molecular pharmacology of the barbiturate site.

Methods

Preparation of membranes

Acetylcholine receptor-rich membranes were prepared from freshly dissected *T. nobiliana* electroplaque by differential and sucrose density gradient centrifugation, as previously described (Dodson *et al.*, 1987). The resultant membranes, which contained 1–2 nmol [³H]-acetylcholine binding sites mg^{-1} protein, were divided into 1 ml aliquots, frozen in liquid nitrogen and stored at -85°C . These aliquots were thawed as needed, stored under nitrogen at 4°C and used within three weeks. Membrane specific activity and results were unaffected by storage conditions.

Measurement of [¹⁴C]-amobarbitone binding to membranes

[¹⁴C]-amobarbitone binding to membranes was determined by a centrifugation assay, as previously described (Dodson *et al.*, 1987). Briefly, membranes (1 μM acetylcholine binding sites) were incubated with [¹⁴C]-amobarbitone (5 μM) and varying concentrations of unlabelled barbiturates in *Torpedo* Ringer solution (composition, mM: NaCl 250, KCl 5, CaCl₂ 3, MgCl₂ 2, Na₂PO₄ 5, pH 7.0) at 25°C for 30 min. Aliquots (100 μl) of the membrane suspension were then transferred in triplicate to microcentrifuge tubes and centrifuged (Beckman Airfuge, Ultracentrifuge 30° rotor, model A-100, 133,000 g , 30 min). After careful aspiration of the resultant supernatant, the pellet was quickly washed three times with 100 μl of ice-cold buffer. To solubilize the pellet, 10% sodium dodecyl sulphate (100 μl) was added to the centrifuge tubes, and the contents transferred to 7 ml glass scintillation vials containing 6 ml of scintillation cocktail. The vials were then kept at 37°C for at least 1 h. After vigorous vortexing, the samples were counted on a

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Table 1. Relative potencies and aqueous solubilities for fourteen barbiturates

Agent	IC ₅₀ ^{a,b} (±s.e.) (μM)	K _I ^{a,b} (±s.e.) (μM)	C _{sat} ^b (mM)
Amobarbitone	18 (1.2)	13 (0.9)	5.0
Aprobarbitone	640 (31)	452 (22)	14.0
Barbitone	3,970 (240)	2,802 (169)	35.0
Butobarbitone	482 (51)	340 (35)	10.0
Allylbarbitone (Butalbarbitone)	827 (58)	584 (41)	6.0
DMBB	365 (30)	258 (21)	1.0
Hexobarbitone			2.0
Mephobarbitone			1.0
Metharbitone			8.0
Methohexitone			1.3
Pentobarbitone	154 (7)	109 (5)	10.0
Phenobarbitone	550 (24)	388 (17)	5.0
Secobarbitone	173 (11)	122 (8)	10.0
Thiopentone	261 (20)	184 (14)	0.7

^a Individual binding parameters were obtained from fitting the combined data of two or more experiments to Equation 1 with $n_H = 1$ and to Equation 2 with $K_d = 12 \mu\text{M}$ (see Results).

^b Concentrations include both the unionized and ionized form of the barbiturate in *Torpedo* Ringer buffer, pH 7.0, at 25°C. DMBB = dimethylbutylbarbituric acid.

Packard Tri-Carb liquid scintillation spectrometer having 98% efficiency. Nondisplaceable binding was defined as binding in the presence of excess amobarbitone (3 mM) and represented approximately 30% of total binding. Maximum displaceable binding was defined as the difference between total and nondisplaceable [¹⁴C]-amobarbitone binding under control conditions in a given experiment.

Data analysis

Values for 50% inhibition of displaceable [¹⁴C]-amobarbitone binding (IC₅₀) were calculated by fitting the data to a logistic function of the form

$$I = (I_{\max} - I_{\min}) \left(\frac{[\text{barb}]^N}{[\text{barb}]^N + \text{IC}_{50}^N} \right) - I_{\min} \quad \text{Eq. (1)}$$

where I is the percentage inhibition for a barbiturate at concentration $[\text{barb}]$, I_{\max} and I_{\min} are the maximum and minimum percentage inhibitions, respectively, and N is the slope parameter corresponding to the Hill coefficient. Apparent inhibition constants (K_I) were calculated from the equation

$$K_I = \frac{\text{IC}_{50} \times K_d}{K_d + \{[\text{C}^{14}\text{]}\text{-amobarbitone}\}} \quad \text{Eq. (2)}$$

where K_d is the previously determined equilibrium dissociation constant for displaceable [¹⁴C]-amobarbitone binding under these conditions ($K_d = 12 \mu\text{M}$, Dodson *et al.*, 1987). Binding data were analysed by an iterative, nonlinear least squares programme on a Macintosh SE computer as previously described (Dodson *et al.*, 1987). Results are expressed as mean ± standard deviation of the combined data of two or more experiments.

Materials

Torpedo nobiliana were purchased from Biofish Associates (Georgetown, MA, U.S.A.). [¹⁴C]-amobarbitone (52 mCi mmol⁻¹) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). [³H]-acetylcholine (0.7–2.0 Ci mmol⁻¹) was obtained from Amersham-Searle (Arlington Heights, IL, U.S.A.). α-Bungarotoxin was purchased from Miami Serpentarium (Miami, FL, U.S.A.). All other materials and drugs were purchased from commercial sources.

Results

Effect of barbiturates on displaceable [¹⁴C]-amobarbitone binding to acetylcholine receptor-rich membranes

Fourteen barbiturates were examined in a self-consistent set of experiments with at least two independent determinations being made for each agent. Ten barbiturates (Table 1) inhibited displaceable [¹⁴C]-amobarbitone binding to acetylcholine receptor-rich membranes, while four had minimal effect. The ten inhibiting barbiturates all appeared to act similarly with typical results shown in Figure 1. Eight of these barbiturates (amobarbitone, aprobarbitone, barbitone, butobarbitone, allylbarbitone, pentobarbitone, phenobarbitone and

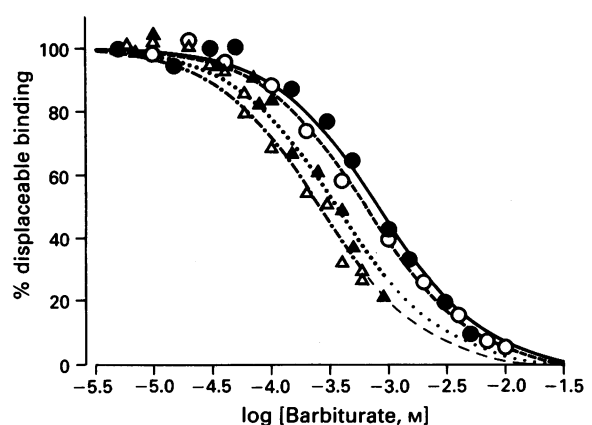


Figure 1 Barbiturate inhibition of displaceable [¹⁴C]-amobarbitone binding: the effect of various concentrations of aprobarbitone (○), allylbarbitone (●), dimethylbutylbarbituric acid (▲) and thiopentone (△) on the displaceable binding of [¹⁴C]-amobarbitone (5 μM) to acetylcholine receptor membranes (1 μM ACh binding sites) was determined by centrifugation assay (see Methods). Maximum displaceable binding was defined as the difference in total binding in the presence and absence of excess amobarbitone (3 mM). For Figures 1–3, free [¹⁴C]-amobarbitone concentrations never varied from total [¹⁴C]-amobarbitone concentrations by more than 5–8%, the results are representative of at least two experiments with each barbiturate and each concentration determined in triplicate (standard deviation of the replicates in all figures was generally 2–5% of the mean and never greater than 10%). The concentration-response curves were drawn using $n_H = 1$ and the IC₅₀ values given in Table 1.

secobarbitone) produced a maximum inhibition (I_{max}) of >90–95%. Thiopentone and DMBB inhibited [14 C]-amobarbitone binding by 75% and 80%, respectively, at the limits of their solubility in buffer (C_{sat} , Table 1). Their low aqueous solubility prevented us from establishing unequivocally their respective I_{max} . However, there was no evidence of a plateau in the percentage inhibition of either barbiturate at C_{sat} . Furthermore, the extrapolated best fit estimates of I_{max} for both barbiturates did not differ within experimental error from 100% inhibition.

Analysis of concentration-inhibition relationships for class one agents

The IC_{50} values for the class one barbiturates were obtained by fitting their respective data to Equation 1, as described in Methods. The Hill coefficient, n_H , ranged from 0.89 to 1.24 with an average standard deviation of 0.063. For purposes of comparison the values of the IC_{50} s given in Table 1 were derived from Equation 1 assuming a common value of $n_H = 1$, but values obtained allowing n_H to vary independently did not differ significantly. The values in Table 1 were then used in Equation 2 to calculate their respective K_I values.

Amobarbitone ($K_I = 13 \mu M$) was the most potent inhibitor of [14 C]-amobarbitone binding. This K_I value was seven fold lower than that of the next most potent barbiturate, pentobarbitone ($K_I = 109 \mu M$). The values for the next eight agents were grouped in a narrow concentration range (100–600 μM) with the rank ordering of pentobarbitone \approx secobarbitone > thiopentone > DMBB > butabarbitalone \approx phenobarbitone > aprobarbitone > allylbarbitone. Although barbitone was also capable of completely inhibiting [14 C]-amobarbitone binding, it was much less potent ($K_I = 2.8$ mM) than the other nine barbiturates.

Analysis of concentration-inhibition relationships for class two agents

Four barbiturates (metharbitone, methohexitone, mephobarbitone and hexobarbitone) had minimal effect on displaceable [14 C]-amobarbitone binding (Figure 2). At their respective C_{sat} s (Table 1), methohexitone, mephobarbitone and hexobarbitone inhibited [14 C]-amobarbitone binding by approximately 30%, 20% and 20%, respectively (Figure 2). Because

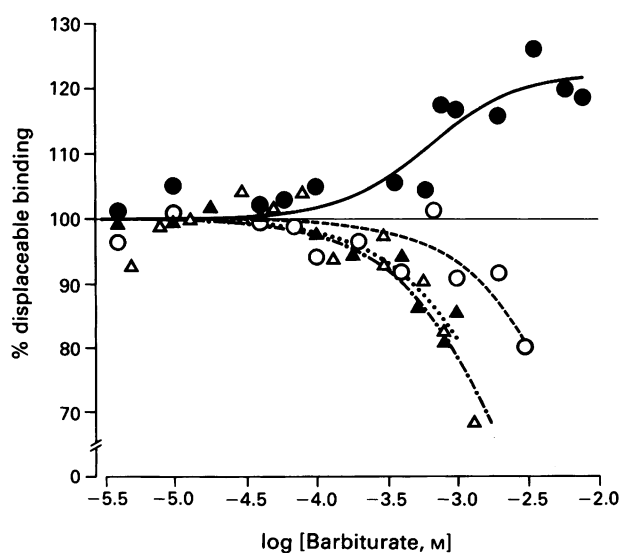


Figure 2 The N-methylated barbiturates are weak modulators of displaceable [14 C]-amobarbitone binding: the concentration-response curves for hexobarbitone (O), mephobarbitone, (Δ), metharbitone (\bullet), and methohexitone (\triangle) were drawn by eye. The experimental conditions were as described in Figure 1.

the magnitude of inhibition was so small at C_{sat} , more detailed analyses could not be undertaken.

Metharbitone (>0.6 mM) was unique in producing a slight, concentration-dependent increase in [14 C]-amobarbitone binding (Figure 2) which apparently plateaued approximately 15–20% above control values at 6–8 mM.

Discussion

This study further characterizes the structural requirements of a [14 C]-amobarbitone binding site on the *Torpedo* acetylcholine receptor. It provides an accurate and self-consistent set of apparent inhibition constants for ten barbiturates and establishes that four others are without substantial effects.

Because the variation in the apparent inhibition constants (K_I s) of the ten class one agents might be accounted for simply by hydrophobicity, or lipid solubility, rather than specific structure-activity relationships, we first compared the K_I s with their octanol/water partition coefficients, $\lambda_{o/w}$ (Table 2). Linear regression yielded a rough correlation between these variables { $\log(K_I)$ versus $\log(\lambda_{o/w})$, $r^2 = 0.49$ } which was in keeping with the observation that the longer the 5' side chain, the higher the barbiturate potency (i.e., secobarbitone > aprobarbitone) (Table 2). However, the value of this correlation is limited, because the slope of the correlation deviated from the expected value of one. For example, amobarbitone and phenobarbitone were approximately 16 and 3.5 times, respectively, more potent, and thiopentone approximately 2.4 times less potent than their predicted values.

Rearrangements of the 5 or 5' chain of a barbiturate also produced results different from those predicted from hydrophobicity. For example, amobarbitone and pentobarbitone are formula isomers, (5-ethyl, 5'(3-methylbutyl) barbituric acid and 5-ethyl,5'(1-methylbutyl) barbituric acid, respectively) with almost identical octanol/water partition coefficients. Yet this simple change in the position of a secondary methyl group made the K_I of pentobarbitone an order of magnitude higher than that of amobarbitone. Similarly, the addition of a methyl to the 1 position of amobarbitone to yield DMBB (5-ethyl, 5'(1,3 dimethylbutyl) barbituric acid) produced the expected increase in the octanol/water partition coefficient but a twenty fold decrease in affinity for the [14 C]-amobarbitone binding site. Similar deviations can also be seen with 5 chain substitutions. Thus, secobarbitone has an allyl group whereas pentobarbitone has an ethyl group on the 5 chain {5-allyl, vs 5-ethyl, 5'(1-methylbutyl) barbituric acid}, yet they have similar binding affinities despite a three fold difference in octanol/water partition coefficients.

Thiopentone differs from pentobarbitone only in having a thiocarbonyl group at the 2 position of the pyrimidine ring instead of a carbonyl group (i.e. 5-ethyl, 5'(1-methylbutyl)-2-thio barbituric acid). Despite enhancing the octanol/water partition coefficient four fold, this substitution decreased the binding affinity slightly.

The most radical changes were produced by substituting a methyl group for a hydrogen on the 1 position of the pyrimidine ring. Although N-methylation increases octanol/water partition coefficients by nearly an order of magnitude (Table 2), all four N-methyl barbiturates examined failed to cause significant displacement of [14 C]-amobarbitone (Table 1 and Figure 2). This loss of potency is not simply a function of the decreased aqueous solubility of the N-methyl analogues (Figure 3), because phenobarbitone and barbitone have IC_{50} s of 0.55 and 2.8 mM respectively, whereas their N-methyl analogues, mephobarbitone and metharbitone, caused little inhibition at twice these concentrations.

Why do the N-methyl barbiturates bind so weakly, if at all, to the amobarbitone site? The simplest explanation is steric hindrance. For example, the fit of the whole pyrimidine ring into a narrow cleft on a binding site could be prevented by

Table 2 Comparison of inhibition constants for the barbiturate site on acetylcholine receptors with other properties

Agent	K_1^a (μM)	Occupancy at [GABA] ^b (%)	[³ H]-BMC ^c (μM)	[³⁵ S]-TBPS ^d (μM)	$\lambda_{o/w}^e$
Amobarbitone	11	0.97			129 ^f
Aprobarbitone	390	0.72			41 ^f
Barbitone	2,371	0.81	> 500		5 ^g
Butabarbitorone	293	0.58			45 ^h
Allylbarbitone	504	0.46			45 ⁱ
DMBB	233	0.14	375	37	177 ⁱ
Hexobarbitone		0		182	110 ^f
Mephobarbitone		0	368	129	107 ^f
Metharbitone		0		125	
Methohexitone		0			3,333 ^h
Pentobarbitone	98	0.57	156	67	135 ^f
Phenobarbitone	246	0.85	195	170	25 ^g
Secobarbitone	108	0.37	143	14	389 ^f
Thiopentone	132	0.10			390 ^j

All data are corrected to the concentration of the unionised form.

^a K_1 , calculated from Table 1 using pKs summarized in Firestone *et al.*, 1986.

^b Occupancy of the barbiturate site at the concentration causing general anaesthesia in tadpoles (Lee-son *et al.*, 1975).

^c [³H]-BMC, concentration causing allosteric inhibition of [³H]-bicuculline methochloride ([³H]-BMC) binding to rat synaptic membranes (Wong *et al.*, 1984; Olsen *et al.*, 1986).

^d [³⁵S]-TBPS, concentration causing inhibition of [³⁵S]-*t*-butylbicyclophosphorothionate (Ticku & Rastogi, 1986).

^e $\lambda_{o/w}$, octanol/water partition coefficient for unionized barbiturates.

Sources: ^f Yih & van Rossum, 1977; ^g Kakemi *et al.*, 1967a,b; ^h Backes *et al.*, 1984; ⁱ Hansch & Anderson, 1967; ^j Korten & Miller, 1979.

N-methyl substitution. Such a mechanism would also explain the lower than expected affinity of thiopentone.

Metharbitone alone of the barbiturates examined increased, rather than decreased, [¹⁴C]-amobarbitone binding, suggesting that it acts by an entirely different mechanism. For example, under our control conditions, approximately 75–80% of the acetylcholine receptors exist in the resting state (Boyd & Cohen, 1980), which has high affinity for amobarbitone, whilst the remainder are in the desensitized state with negligible affinity for amobarbitone (Dodson *et al.*, 1987). Thus, metharbitone could act by converting all of the acetylcholine receptors into the resting state, perhaps by binding to a separate allosteric site.

The nicotinic and GABA receptors belong to the same superfamily, having high sequence homology especially in their transmembrane regions (Schofield *et al.*, 1987; Barnard *et al.*, 1988). Although specific barbiturate binding has not been directly demonstrated on the GABA receptor (because of the low density of binding sites), there are extensive parallels between the barbiturate interactions with the two receptors

(Barker *et al.*, 1980; Skolnick *et al.*, 1982; Heidmann *et al.*, 1983; Olsen *et al.*, 1986; Ticku & Rastogi, 1986; Dodson *et al.*, 1987; Schwartz, 1988).

The affinity of barbiturates for a possible allosteric site on the GABA receptor has been inferred from studies of their effects either on the binding of the picrotoxin-like caged convulsant [³⁵S]-*t*-butylbicyclophosphorothionate ([³⁵S]-TBPS) to its allosteric site or on the binding of the GABA antagonist [³H]-bicuculline methochloride ([³H]-BMC) (sources and results of these studies are given in Table 2). Although the affinity for the barbiturate site on both receptors is similar for our class one agents, the N-methylated barbiturates discriminate strongly between them. Thus, barbiturate sites on the ACh and the GABA receptors are similar but not identical, a conclusion also reached by Roth *et al.* (1989). They found that whilst the (+)-pentobarbitone enantiomer had equal apparent affinity for both receptors, the enantiomers of pentobarbitone bound to the acetylcholine receptor with the reverse stereoselectivity of that reported by Ticku & Rastogi (1986) for the GABA receptor. Another difference was that [¹⁴C]-amobarbitone inhibition curves of group one agents on the acetylcholine receptors had a uniform slope (i.e., $n_H = 1$, Figure 1), whereas action on the GABA receptor exhibited a wide range of Hill coefficients (Leeb-Lundberg & Olsen, 1982).

The barbiturate site on the acetylcholine receptor does not appear to be specifically involved in general anaesthesia (Dodson *et al.*, 1987). For example, hexobarbitone, mephobarbitone and methohexitone are all potent anaesthetics (Table 2), yet barely inhibit [¹⁴C]-amobarbitone binding (Figure 2). Even after excluding the N-methyl barbiturates, which may be rapidly demethylated *in vivo* (Butler, 1953), the rank order for displacing [¹⁴C]-amobarbitone still differs markedly from that for inducing anaesthesia. This does not preclude a possible connection with a more subtle measure of anaesthetic potency, such as amnesia. Indeed, there may be a nicotinic component in the neuronal processes involving memory (Levin *et al.*, 1989).

In conclusion, the fourteen diverse barbiturates surveyed could be categorized into two distinct classes according to their ability to displace [¹⁴C]-amobarbitone from its allosteric site on the *Torpedo* acetylcholine receptor. Ten of the barbiturates could be classed as competitive inhibitors of [¹⁴C]-amobarbitone binding. However, a second class of four barbiturates had only minor effects (<30%) on [¹⁴C]-amobarbitone binding. All of this second group of barbiturates

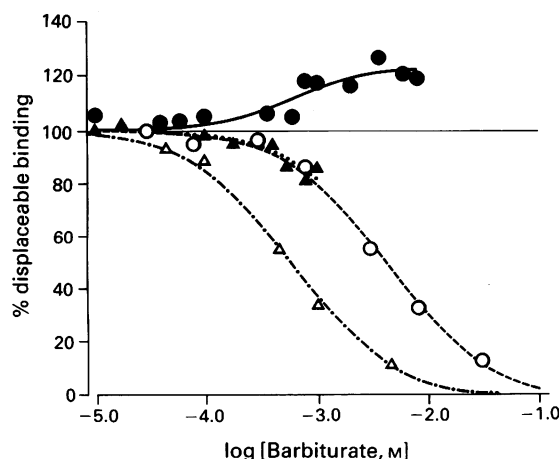


Figure 3 Comparison of phenobarbitone and barbitone with their N-methylated analogues: the effect of various concentrations of phenobarbitone (Δ) and barbitone (\circ) and their respective N-methyl analogues, mephobarbitone (\blacktriangle) and metharbitone (\bullet), on displaceable [¹⁴C]-amobarbitone binding. See Figures 1 and 2.

were N-methylated on the 1 position of the pyrimidine ring. These results support the existence of a barbiturate binding site on the acetylcholine receptor with specific structural requirements for binding that are different from those predicted from simple hydrophobic interactions.

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